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Enzyme-immunoassay for the measurement of antigens using peroxidase conjugates.

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Summary. — Human and rat IgG were labelled with peroxidase. Procedures were established using these conjugates and insoluble antibody immunoadsorbents for the quantitation of humoral IgG. The basic principles of these procedures are those already developed for quantitative radio-immunoassay, enzyme activity measurement substituting radioactivity counting. The procedures allowed the determination of 10-200 ngs of antigen. The values obtained in unknown samples using these enzyme-immunoassays were in good agreement with those obtained using the quantitative single radial immunodiffusion technique.

INTRODUCTION.

The use of enzyme-labelled antigens or antibodies for the light or electron microscopic localization of cellular constituents has been reported [1]. According to this procedure, the enzyme-labelled protein is allowed to react with the cellular antigen or antibody and then the sites of bound enzyme are revealed with appropriate cytochemical staining techniques.

Because the quantity of an enzyme can be accurately determined by appropriate enzymological techniques, enzyme-labelled antibodies can also be used to quantitate cellular antigens [2].

Similarly, enzyme-labelled antigens can be used for the quantitation of humoral antigens [3, 4, 5, 6]. In this case, the basic principles of the method employed are those developed for quantitative radio-immunoassay. Thus, the method involves incubation of a determined quantity of enzyme-labelled antigen with increasing concentration of unlabelled one, in the presence of a given amount of antibody directed against this antigen. The extent of binding of the enzyme-labelled antigen in the presence of known varying amounts of unlabelled antigen allows the establishment of a reference curve from which unknown concentrations of the antigen in samples can be determined.

In the present paper, we describe, in detail, the procedures and the conditions required to measure small quantities of humoral antigens with peroxidase-labelled antigens. The values obtained in unknown samples using the present enzyme-immunoassay are in good agreement with those obtained using the quantitative single radial immuno-diffusion technique [7].

MATERIALS AND METHODS.

Preparation of human and rat IgG.

Human IgG and rat IgG (IgG_A, IgG_B) were isolated according to published procedures from normal pooled sera using ammonium sulfate precipation and diethylamino-ethyl cellulose chromatography [8]. Immunoelectrophoresis of the preparations using homologous anti-whole serum protein antisera revealed of precipitation corresponding only to the IgG family.

Preparation of antisera and isolation of antibodies.

Rabbit anti-human and anti-rat IgG and sheep anti-rabbit IgG and anti-rat IgG sera were prepared following immunization schedules described elsewhere [8 9]. The antisera revealed only IgG when tested with the corresponding normal sera by immunoelectrophoresis Antibodies were isolated by passage of the whole sera on immunoadsorbents prepared by co-polymerization at pH 5 of 4 parts bovine serum albumin and one part IgG [10].

Insolubilization of antisera and of isolated antibodies.

Whole sheep or rabbit antisera were diluted to 30 mg protein per ml with 0.2 M acetate buffer pH 5 and then polymerized with ethylchloroformiate [11].

Ammonium sulfate precipitated γ-globulins from antisera or specifically isolated antibodies were coupled to glutaraldehyde treated Bio-Gel P-300 minus 400 mesh following procedures already described [12].

The insoluble antibody derivatives were suspended in sterile physiological saline and stored at 4°C until used. The antibody concentration in the suspension was 0.5 to 1 mg/ml.

Assay of peroxidase activity.

Peroxidase activity was measured by using H_2O_2 as the substrate and o-dianisidine as the hydrogen donor [13]. The reagents were added to 0.6 ml of 1 M phosphate buffer pH 6 in the following order : 0.6 ml of a 0.3 p. cent aqueous solution of H_2O_2 ; 58.8 ml of distilled water ; 0.5 ml of a 1 p. cent solution of o-dianisidine in methanol with continuous stirring.

To measure peroxidase activity, 0.1 ml of the enzyme solution containing 1 to 5 ngs of peroxidase were added to 2.9 ml of the substrate solution and the reaction mixture was allowed to incubate at room temperature (25°C). After 1 hour, the reaction was stopped with 1 drop of 5N HCl with thorough mixing on a Vortex vibrator and the color development was read at 400 mμ.

Dilution medium.

All dilutions were made with a sterile solution of 0.1 M phosphate buffer pH 6.8 containing 1 p. cent bovine serum albumin.

Labelling of human and rat IgG with peroxidase.

Peroxidase was coupled to IgG following a procedure recently described [14]. Twenty mgs of peroxidase were dissolved in 0.3 ml 0.1 M phosphate buffer pH 6.8 containing 1.25 per cent glutaraldehyde (TAAB Lab. Reading, England). The solution was allowed to stand for 18 hrs at room temperature and was then filtered through a Sephadex G-25 column (70 × 0.9 cm) equilibrated with 0.15 M NaCl in order to remove excess of unreacted glutaraldehyde. The fractions containing the activated peroxidase were pooled (final volume about 3 ml) ; 1 ml of 0.15 M NaCl containing 10 mg of IgG was added, followed by the addition of 0.2 ml of 1 M carbonate - bicarbonate buffer pH 9.5. After 24 hours at 4°C, the reaction mixture was dialysed at 4°C overnight against 0.15 NaCl, buffered at pH 7.4 and then stored at 4°C for one week before subsequent use. When employing these preparations for the determination of IgG, it is necessary to eliminate free peroxidase as well as non-labelled IgG from the conjugate. To do this, the reaction mixture was chromatographed at 4°C on a Sephadex G-200 column (100 × 2.6 cm) equilibrated with 0.15 M NaCl buffered to pH 7.4. The protein content of the fractions was read at 280 mμ and that of peroxidase at 403 mμ. The first peak eluting from the column contained only IgG labelled with peroxidase [14]. The fractions of the first peak were pooled and read at 280 and 403 mμ to determine the peroxidase-labelled IgG concentration. Under the above described conditions, the concentrations varied between 0.080 and 0.100 mg/ml.

After filtration through a sterile Millipore filter 0.22 μ, glycerol was added to a final concentration of 30 p. cent and the solution was distributed in sterile aliquots of 0.2 ml and stored either at 4°C or at — 20°C. Prior to use, 0.1 ml of the labelled IgG was diluted to give a peroxidase activity (0.1 ml sample) corresponding to an optical density at 400 mμ of 4.00 to 6.00.

RESULTS AND DISCUSSION.

Direct method for the determination of IgG.

To measure rat IgG using insoluble anti-rat IgG antibody, one has first to establish the optimal amount of insoluble antibody to be used. This was done by adding increasing amounts of immunoadsorbent suspension to a series of duplicate tubes to give a final concentration of insoluble antibody ranging from 0.050 to 500 μg per ml. The tubes were then centrifuged for 10 minutes at 3.500 g and the supernatants discarded. 0.1 of peroxidase-labelled IgG (peroxidase activity corresponding to an optical density of 4.0 to 6.0) was

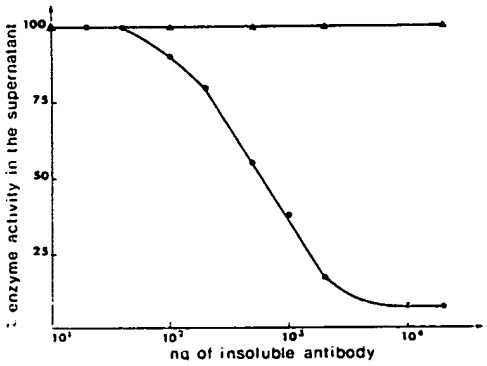

FIG. 1. — *Direct method* : Binding of peroxidase-labelled rat IgG (●——●) and of peroxidase-labelled human IgG (▲ ··▲) by increasing amounts of insoluble rabbit anti-rat Ig serum. The insoluble antiserum was prepared with ethylchloroformiate.

then added to the tubes and the total volume was made up to 1 ml. The tubes were stoppered and placed on a rotating plate inclined at about 60°. After 2 hours incubation with constant gentle rotating agitation at room temperature, the tubes were centrifuged and peroxidase activity was determined in 0.1 ml of the supernatant (fig. 1). From the curve in figure 1, the amount of insoluble antibody binding 40 to 60 p. cent of the peroxidase-labelled IgG was chosen.

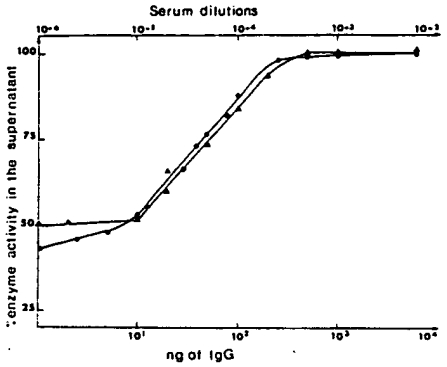

FIG. 2. — *Direct method* : Inhibition binding curves of peroxidase labelled rat IgG by standard quantities of rat IgG (●——●) and by dilutions of normal rat serum (▲——▲). The same immunoadsorbent as in Fig. 1 was used. Mean values of two experiments.

To measure IgG, 1 to 10^4 ngs of unlabelled IgG in 0.1 ml of the dilution medium were added to a series of duplicate tubes containing the previously determined amount of insoluble antibody (0,3 ml). The tubes were agitated for 2 hours at room temperature and then 0.1 ml of the peroxidase labelled IgG was added. The total volume was brought to 1 ml with diluting medium and incubation with rotating agitation was allowed to proceed for 16 hours at 4°C. The tubes were then centrifuged and the peroxidase activity was determined in 0.1 ml of the supernatants.

The curves obtained with known and unknown quantities of unlabelled IgG are shown in figure 2 ; it is evident that with this procedure quantities ranging from 20 to 200 ngs of IgG can be measured. The procedure was established after a set of trials where the dose of labelled antigen, the incubation time and the temperature were varied. Optimal conditions were definied from the different results obtained.

Indirect method for the determination of IgG.

According to this procedure, labelled and unlabelled antigen were first allowed to react with soluble antiserum directed against this antigen and the antigen-antibody complexes were then precipitated with insoluble antibody directed against the immunoglobulins of the antiserum.

The following refers to the measurement of rat IgG using rabbit antiserum anti-rat IgG and insoluble sheep antiserum antirabbit IgG but the sames holds true for the measurement of human IgG.

A sufficiently sensitive assay can only be obtained when the soluble antisera are used at dilutions higher than 1 : 1000. Consequently, in the first step of the procedure, one has to establish the amount of insoluble antibody required to precipitate all the peroxidase-labelled IgG fixed by the rabbit antibody present in the diluted antiserum. This was done by adding 0.1 ml of peroxidase-labelled IgG (peroxidase activity corresponding to an optical density of 4.0 to 6.0) and 0.5 ml of rabbit antiserum anti-rat IgG diluted 1.000 fold to a series of duplicate tubes and incubating them at room temperature for 30 minutes.

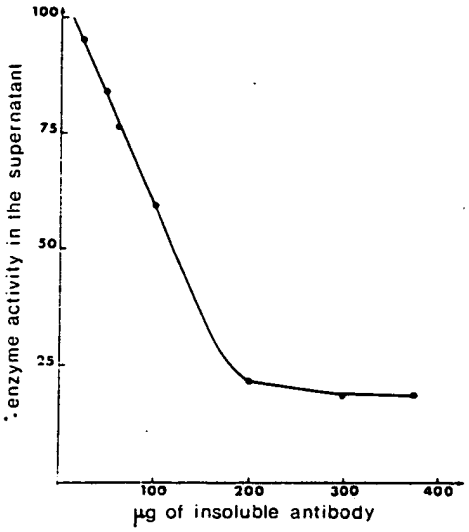

FIG. 3. — *Indirect method* : Binding of (peroxidase-labelled rat IgG)/(rabbit anti-rat IgG antibody) immune complexes by varying amounts of sheep anti-rabbit IgG immunoadsorbent. The rabbit anti-rat IgG antiserum was used at 1 : 1,000 dilution. Purified sheep anti-rabbit IgG antibody was coupled to polyacrylamide beads and was employed as the immunoadsorbent.

Increasing amounts of sheep anti-rabbit IgG immunoadsorbent were then added (final concentration of insoluble antibody from 0.050 to 0.5 mg/ml) and the final volume was brought to 1 ml. The tubes were then incubated, under rotating agitation, for 2 hours at room temperature, centrifuged

and finally the peroxidase activity was determined in 0.1 ml of the supernatant (fig. 3). Amounts of insoluble antibody corresponding to the plateau of the curve should be used. In this way, the amount of immunoadsorbent which should always be in excess and which precipitates all the labelled IgG fixed by the antibody can be determined.

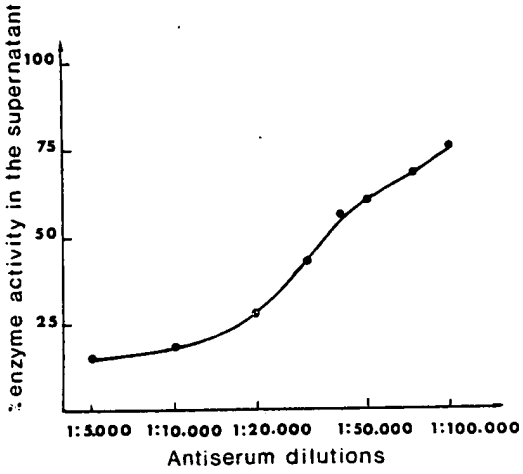

FIG. 4. — *Indirect method* : Binding of peroxidase-labelled rat IgG by rabbit anti-rat IgG antibody as a function of the antiserum dilution employed. The same reagents as in figure 3 were used.

In the second step of the procedure, the optimal dilution of the rabbit anti-rat IgG antiserum has to be established. For this, a series of duplicate tubes containing 0.1 ml of peroxidase-labelled IgG and 0.5 ml of rabbit antiserum anti-rat IgG diluted from 1,000 to 50,000 times were incubated at room

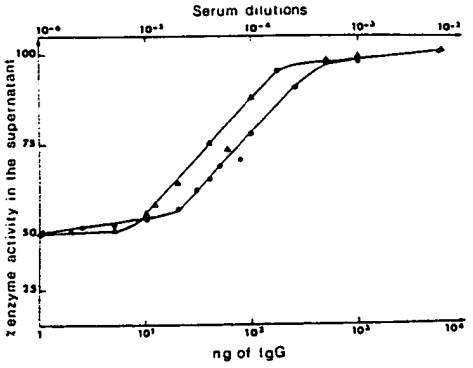

FIG. 5. — *Indirect method* : Inhibition binding curves of peroxidase labelled rat IgG by standard quantities of rat IgG (●- - -●) and by dilutions of normal rat serum (▲—▲). The same reagents as in figure 3 were used. Mean values of two experiments.

temperature for 30 minutes ; then the optimal amount of insoluble anti-rabbit IgG antibody previously determined was added and the final volume was brought to 1 ml. Incubation of this suspension, centrifugation and determination of enzymatic activity were then carried out as described above. A curve was plotted by using different dilutions of antisera (fig. 4) and, from this curve, a dilution of antiserum sufficient to bind 30 to 50 p. cent of the peroxidase labelled IgG was chosen. To measure IgG, 1 to 10^4 ngs of unlabelled IgG in 0.1 ml of the diluting medium were added to a series of duplicate tubes containing 0.5 ml of the previously determined dilution of the antiserum. The tubes were incubated at room temperature for 30 minutes, then 0.1 ml of the labelled IgG was added and the solutions were incubated at room temperature for an additional 30 minutes. After this time, the previously determined quantity of insoluble antibody was added and the suspensions were then incubated under totation for 2

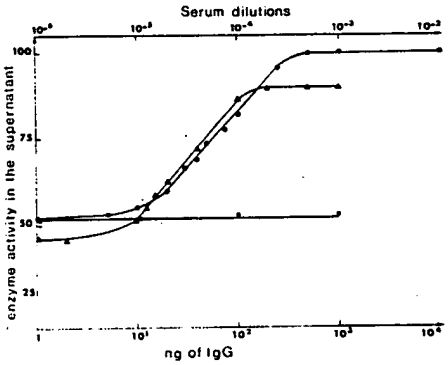

FIG. 6. — *Indirect method* : Inhibition binding curves of peroxidase-labelled human IgG by standard quantities of human IgG (●—●), rat IgG (■—■) and by dilutions of normal human serum (▲—▲).

hours et room temperature. Subsequent centrifugation and determination of enzymatic activity were performed as before.

Curves obtained with known and unknown quantities of rat IgG are shown in figure 5. It is evident that by this procedure, amounts ranging from 10 to 200 ngs of IgG can be quantitated. Similar results were obtained when the procedure was employed for the measurement of human IgG (fig. 6). As for the direct method, the present procedure was established after a set of trials where the quantity of labelled antigen, time and temperature of incubation were varied.

GENERAL CONSIDERATIONS.

In the above experiments, all dilutions were made with sterile solutions of 0.1 M phosphate buffer pH 6.8 containing 1 p. cent bovine serum albumin. The presence of bovine serum albumin at all stages was necessary to avoid an eventual non specific adsorption of labelled antigen by immunoadsorbents and to stabilize the catalytic activity of peroxidase. However, care should be paid to the albumin employed. Indeed with one lot of albumin, a 50 p. cent decrease of peroxidase activity was observed, perhaps indicating that some albumin preparations contain peroxidase inhibitor(s). In this connection, it is to be stressed that inhibitors of microbial growth such as sodium azide or merthiolate should not be used for the preservation of reagents employed in these procedures because these substances are also effective inhibitors of peroxidase. Peroxidase labelled antigen and insoluble antibody when kept at + 4°C under sterile conditions can be used for months without any appreciable loss of biological activity.

From these results, it appears that ethylchloroformiate antibody immunoadsorbents and acrylamide bead antibody immunoadsorbents were equally effective in the determination of antigens. Non specific adsorption of heterologous labelled antigen by these immunoadsorbents could not be detected (see also figure 2). The only exception was with sheep antisera insolubilized with ethyl-chloroformiate, where a substantial non specific adsorption was observed. For this reason use of sheep antiserum insolubilized with ethylchloroformiate has to be avoided.

Both direct and indirect methods were found to be highly reproducible. A series of experiments using different lots of peroxidase labelled antigen and insoluble antibody were repeated in a period of 10 months and each time almost identical results were obtained. The procedures can be used to determine amounts of IgG ranging from 10 to 200 ngs. As in quantitative radio-immunoassay the sensitivity of the procedures can be increased by choosing a dilution of antibody that binds less than 50 p. cent of the labelled antigen. However, in this case the slope of the surve decreases and consequently, the measurement of the antigen is determined less precisely. Heterologous antigens were not found to inhibit significantly the binding of peroxidase labelled IgG with its homologous antibody (see figure 6), indicating the high specificity of the procedures employed.

Unknown samples of IgG present in normal human or rat sera were analysed by the present immunoassay procedures and by quantitative single radial immunodiffusion techniques [7]. In all cases, good agreement between the values obtained by the two techniques was noted. Thus for example, three different measurements of rat IgG performed in the same serum by the indirect enzyme immunoassay gave a mean value of 15 mg of IgG per ml and by radial immunodiffusion, 12.9 mg/ml. The same experiments gave for normal human serum a value of 12 mg of IgG per ml both with the enzyme immunoassay and the radial immunodiffusion.

The indirect method seems to possess certain advantages when compared to the direct one : Four hours are sufficient to complete the experiments when the indirect method is employed while 24 hours are needed with the direct one. Better duplicate measurements are obtained with the indirect method because it employs soluble antibody which can be accurately determined in the competition experiments. The direct method requires insoluble antibody which cannot always be determined accurately. Thus low quantities of antigen can be more precisely determined with the indirect method.

The enzyme-immunoassay procedures as developed here for the measurement of IgG are as sensitive as the quantitative radio-immunoassay which also uses insoluble antibody [15]. However, peroxidase labelled antigen can be used for at least 6 months while antigen labelled with radioactive iodine can not be used for long periods of time and should measured and readjusted each time. Non specific adsorption of peroxidase-labelled antigen is almost non existant while high non specific absorption is often reported with radioimmunoassay techniques. Consequently, the interpretation of the results might be more objective when enzyme immunoassay rather than quantitative radio-immunoassay is used.

RÉSUMÉ.

Les IgG humaines et de rat ont été marquées avec la peroxydase. Les techniques décrites utilisent ces conjugués et des anticorps insolubilisés pour le dosage des IgG humorales. Les principes de base de ces techniques sont ceux déjà développés pour la radioimmunologie quantitative, la mesure de l'activité enzymatique remplaçant le comptage de la radioactivité. Avec ces techniques, nous avons pu mesurer des quantités de l'ordre de 10 à 200 ng dans des échantillons de sérum dont la quantité en IgG était inconnue. Ces valeurs sont en accord avec celles obtenues par la technique d'immunodiffusion quantitative radiale.

REFERENCES.

1. Avrameas, S. (1970) *Int. Rev. Cytol.*, 27, 349.
2. Avrameas, S. & Guilbert, B. (1971) *Europ. J. Immunol.*, 1, 396.
3. Avrameas, S. & Guilbert, B. (1971) *Compt. Rend. Acad. Sci.*, 273, 2705.
4. Engvali, E. & Perlmann, P. (1971) *Immunochemistry*, 8, 871.
5. Engvall, E., Jonsson, K. & Perlmann, P. (1971) *Biochem. Biophys. Acta*, 251, 427.
6. Van Weemen, B. K. & Schuurs, A. H. W. M. (1971) *FEBS Letters*, 15, 232.
7. Mancini, G., Carbonara, A. O. & Heremans, J. F. (1965) *Immunochemistry*, 2, 235.
8. Gonatas, N. K., Antoine, J. C., Stieber, A. & Avrameas, S. (1972) *Laboratory Invest.*, 26, 253.
9. Avrameas, S., Taudou, B. & Ternynck, T. (1971) *Int. Arch. Allergy*, 40, 161.
10. Avrameas, S. & Ternynck, T. (1969) *Immunochemistry*, 6, 53.
11. Avrameas, S. & Ternynck, T. (1967) *J. Biol. Chem.*, 242, 1651.
12. Ternynck, T. & Avrameas, S. (1972) *FEBS Letters*, 23, 24.
13. Bergmeyer, H. U. (1963) *in* « Methods of Enzymatic Analysis » ; Verlag Chemie, Weinheim, and Academic Press, New York.
14. Avrameas, S. & Ternynck, T. (1971) *Immunochemistry*, 8, 1175.
15. Mann, D., Granger, H. and Fahey, J. L. (1969) *J. Immunol.*, 102, 618.